REMARKS/ARGUMENTS

With this amendment, claims 1-2, 4, 6-7, 9, 11-17, 19-24, and 38 are pending. Claim 18 is withdrawn. Claims 3, 5, 8, 10, and 25-37 are cancelled without prejudice to subsequent revival. New claim 38 is added. For convenience, the Examiner's rejections are addressed in the order presented in the February 22, 2007 Office Action.

Applicants thank Examiner Kaushal for assistance to resolve a mistake in response to the restriction requirement. Applicants elected group II drawn to a method of identifying a compound that modulates angiogenesis using a SUSP-1 polypeptide, but referred to the SUSP-1 polypeptide as SEQ ID NO:456 in the response. SEQ ID NO:456 is a SUSP-1 nucleic acid sequence. In an interview on May 10, 2007 with Applicants' representative Beth Kelly, Examiner Kaushal agreed to proceed with prosecution of claims that recite SEQ ID NO:457, the amino acid sequence of the SUSP-1 protein.

I. Status of the claims

Claims 1 and 24 are amended to recite contacting a test compound with a SUMO protease (SUSP-1) polypeptide that has 95% identity to SEQ ID NO:457 and that regulates angiogenesis in an endothelial cell. Support for these amendments is found throughout the specification, for example, at page 29, lines 26-34 and at page 69, lines 19-32. Claims 6, 11 and 24 are amended to recite that the SUSP-1 protein has protease activity. Support for these amendments is found throughout the specification, for example, at page 17, line 32. New claim 38 is added and recites that the compound is an siRNA molecule that inhibits expression of a nucleic acid that encodes the SUSP-1 polypeptide in a host cell. These amendments add no new matter.

II. Rejections under 35 U.S.C. §112, first paragraph, written description

Claims 1-17 and 19-24 are rejected under 35 U.S.C. §112, first paragraph for allegedly failing to comply with the written description requirement. The Office Action alleges that those of skill would not recognize that the inventors had possession of the claimed invention

at the time of filing. The claims are now amended to recite SUSP-1 polypeptides that have 95% identity to a reference sequence and that regulate angiogenesis in endothelial cells, and/or have protease activity. To the extent the rejection applies to the amended claims, Applicants respectfully traverse the rejection.

As currently applied, the specification does comply with US patent law for description of a nucleic acid or amino acid sequence. The Federal Circuit court of Appeals addressed the description adequate to show one of skill that the inventors were in possession of a claimed genus at the time of filing. *See, e.g., Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 63 USPQ2d 1609 (Fed. Cir. 2002). An applicant may also show that an invention is complete by

> . . . disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention . . . *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. *Id.* at 1613.

Furthermore, "description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces." *See, e.g.*, 66 Fed. Reg. 1099, 1106 (2001).

Functional assays to identify SUSP-1 polypeptides, as recited in the claims, were known to those of skill and are disclosed in the specification. Assays for regulation of angiogenesis by SUSP-1 assays are disclosed in the specification at, *e.g.*, page 69, lines 19-32. SUSP-1 protein was known to have protease activity at the time of filing. *See, e.g.*, Kim *et al. J. Biol. Chem.* 275:14102-14106 (2000), submitted as Exhibit A.

Applicants direct the Examiner's attention to Example 14 of the Synopsis of Application of Written Description Guidelines which analyzes a claim directed to a protein having an amino acid sequence at least 95% identical to SEQ ID NO:3 and that has a specific activity. In these Guidelines, the Patent Office concluded that the claim was adequately described within the meaning of 35 U.S.C. §112, first paragraph. The SUSP-1 protein does have protease activity as discussed in the specification at, *e.g.*, at page 17, line 32. Therefore, on the

basis of Written Description Guidelines issued by the USPTO, the present claims directed to SUSP-1 polypeptides that are 95% identical to SEQ ID NO:457, meet the written description requirement.

In view of the above arguments and amendments, withdrawal of the rejection for alleged lack of written description is respectfully requested.

III. Rejections under 35 U.S.C. §112, first paragraph, enablement

Claims 1-17 and 19-24 are rejected because the specification as filed allegedly does not enable the claims. According to the Office Action, undue experimentation is required to practice the claimed invention. The Office Action does indicate that the specification enables a method for identifying a compound that modulates $\alpha v\beta 3$ expression by modulating SUSP-1 activity. *See, e.g.*, Office Action at page 6. Applicants note that the specification provides experimental evidence that modulation of SUSP-1 activity also modulates tube formation by endothelial cells. See, *e.g.*, specification at page 69, lines 29-32.

Factors such as the amount of guidance presented in the specification and the presence of working examples must be considered to determine whether undue experimentation is required to practice the claimed invention (*see*, *Ex Parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int. 1985) and *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988)). As described in *Wands*, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed" (*see*, *Wands*, USPQ2d at 1404, quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982)). Moreover, "[a] patent need not teach, and preferably omits, what is well known in the art." MPEP 2164.01 *citing In re Buchner*, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481, 489 (Fed. Cir. 1984).

As set forth in the Manual of Patent Examining Procedure (MPEP) § 2164.01, "the test of enablement is not whether any experimentation is necessary, but whether... it is undue." Further, the "fact that experimentation may be complex does not necessarily make it

undue, if the art typically engages in such experimentation" (citations omitted). Finally, claims reading on inoperative embodiments are enabled if the skilled artisan understands how to avoid inoperative embodiments. *See, e.g., In re Cook and Merigold*, 169 USPQ 299, 301 (C.C.P.A. 1971).

The specification provides examples of at least two assays that can be used to assay the angiogenesis regulatory function of an SUSP-1 protein. SUSP-1 angiogenesis regulatory activity is demonstrated in an $\alpha v\beta 3$ expression assay and in a tube formation assay as disclosed at page 69, lines 19-32. As discussed above, the protease activity of SUSP-1 was known at the time of filing, *e.g.*, as disclosed in Kim *et al. J. Biol. Chem.* 275:14102-14106 (2000). In order to establish a prima facie case of lack of enablement, the Examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). The Examiner has not provided any reasoning to suggest that those of skill would not be able to identify a functional SUSP-1 protein with the recited functional characteristics, *e.g.*, ability to regulate angiogenesis and protease activity.

In view of the above arguments and amendments, withdrawal of the rejection for alleged lack of enablement is respectfully requested.

IV. Rejections under 35 U.S.C. §112, second paragraph

Claims 1-17 and 19-24 are rejected for allegedly being indefinite. First, various claims are rejected for recitation of the terms "functional effect", "physical effect", and "chemical effect." In order to expedite prosecution, those terms have been deleted from the claims. Claims 6 and 11 were rejected for recitation of the term "enzymatic activity." In order the expedite prosecution, claims 6 and 11 now recite protease activity. Claims 4 and 9 are amended to recite measuring ligand or substrate binding to the SUSP-1 polypeptide. As SUSP-1 is a SUMO protease, those of skill can identify the ligand or substrate, *i.e.*, SUSP-1, without difficulty.

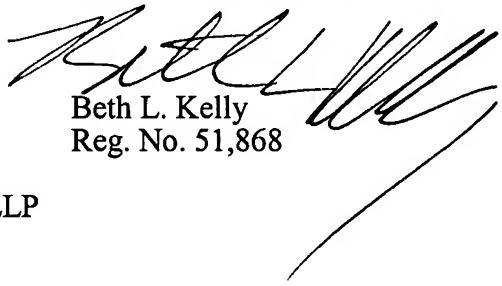
In view of the above arguments and amendments, withdrawal of the rejection for alleged indefiniteness is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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A New SUMO-1-specific Protease, SUSP1, That Is Highly Expressed in Reproductive Organs*

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A full-length cDNA encoding a SUMO-1-specific protease, named SUSP1, was identified and cloned for the first time from the human brain. Nucleotide sequence analysis of the cDNA containing an open reading frame of 3336 base pairs revealed that the protease consists of 1112 amino acids with a calculated molecular mass of 126,116 Da. Like yeast Ulp1, SUSP1 is a cysteine protease containing the well conserved His/Asp/Cys catalytic triad. SUSP1 expressed in *Escherichia coli* cells efficiently released SUMO-1 from SUMO-1·β-galactosidase fusion but not from other ubiquitin-like protein fusions, including Smt3·β-galactosidase, suggesting its role in the generation of matured SUMO-1 specifically from its precursors. Interestingly, reproductive organs, such as testis, ovary, and prostate, contained much higher amounts of SUSP1 mRNA than colon and peripheral blood leukocyte, whereas other tissues, such as heart and spleen, had little or none. In addition, confocal microscopy using green fluorescent protein·SUSP1 fusion showed that SUSP1 is exclusively localized to the cytoplasm of NIH3T3 and HeLa cells. These results suggest that SUSP1 may play a role in the regulation of SUMO-1-mediated cellular processes particularly related to reproduction.

Ubiquitin (Ub)[1] is a highly conserved 76-amino acid polypeptide, which is involved in a variety of cellular processes, including regulation of intracellular protein breakdown, cell cycle regulation, signal transduction, transcription, and antigen presentation (1–3). This small protein is covalently ligated to a variety of target proteins through the action of a multi-enzyme system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes (3–4). The proteins ligated to multiple units of Ub are then degraded by the 26 S proteasome (5). Recently, a number of other small molecules, so called Ub-like molecules (Ubls), have been identified (6, 7). These proteins are structurally related to Ub and can be ligated to target proteins in a similar manner with Ub (8). However, covalent attachment of Ubls does not result in degradation of the modified proteins (6–8). To date, several Ubls, such as SUMO-1/Smt3, NEDD8/Rub1, UCRP, and Fub, have been identified (6–8). Of these, the best characterized Ubl is the mammalian SUMO-1 (also called UBL1, sentrin, PIC1, GMP1, or SMT3c), which can be conjugated to a variety of cellular proteins, such as promyelocytic leukemia protein, RanGTPase-activating protein (RanGAP1), and inhibitor of nuclear factor-κB (IκBα) (9–11). SUMO-1 modification is implicated in the targeting of RanGAP1 to the nuclear pore complex (9, 10) as well as in stabilization of IκBα from degradation by the 26 S proteasome (11).

Like Ub, all of the Ubls are synthesized as precursor proteins with one or more amino acids following the C-terminal Gly-Gly residues of the mature Ubl proteins (6, 7). Thus, the tail sequences of the Ubl precursors need to be removed by Ubl-specific proteases (Ulps) prior to their conjugation to target proteins. Li and Hochstrasser (12) have recently identified and cloned an Ubl-specific protease, called Ulp1 in yeast, which can generate the mature form of Smt3 and SUMO-1. Ulp1 shows no sequence similarity to any known deubiquitinating enzymes. Interestingly, this protease is required for G_2/M phase progression of the cell cycle.

Recently, we have identified a new 30-kDa SUMO-1 hydrolase from extracts of bovine brain, which is different from the 72-kDa yeast Ulp1 (13). This enzyme can generate free SUMO-1 molecules not only from a SUMO-1·peptide fusion but also from RanGAP1·SUMO-1 conjugates, suggesting that the removal of SUMO-1 from its protein conjugates, like deubiquitination, may play an important role in regulation of SUMO-1-mediated cellular processes. In addition, a computer-assisted homology search in the cDNA data base reveals the existence of many different putative proteins having a conserved presumptive catalytic domain homologous to yeast Ulp1. Thus, it appears that a family of SUMO-1-specific proteases that are structurally related is universally distributed in a wide variety of eukaryotic cells. Here we report cloning and characterization of a new SUMO-1-specific protease, named as SUSP1, which is highly expressed in human reproductive organs.

EXPERIMENTAL PROCEDURES

DNA Manipulation—The KIAA0797 cDNA clone (GenBank™ accession number AB018340), which contains a conserved putative catalytic domain homologous to yeast Ulp1 (12), was kindly provided by Dr. Takahiro Nagase (Kazusa DNA Research Institute, Japan). The 4128-

* This work was supported by grants from the Korea Science and Engineering Foundation through the (BK Program Research Center for Cell Differentiation, Korea Ministry of Education, and CREST, Japan Science and Technology Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF196304.

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[1] The abbreviations used are: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Ubls, Ub-like molecules; RanGAP1, Ran-GTPase-activating protein; IκBα, inhibitor of nuclear factor-κB; Ulp, Ubl-specific protease; SUSP, SUMO-1-specific protease; PCR, polymerase chain reaction; PESTc, MHISPPEPESEEEEEHYC; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

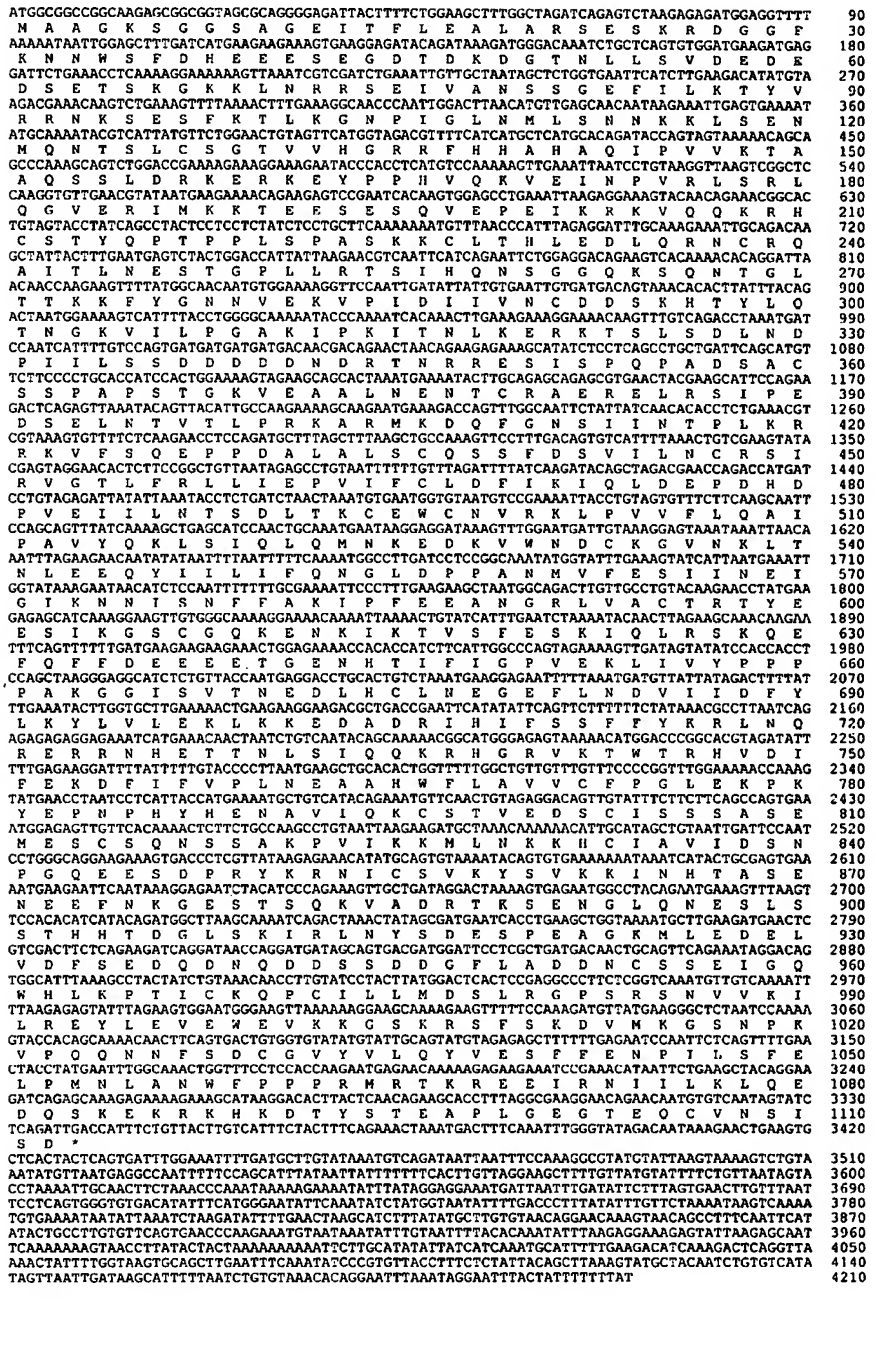

FIG. 1. **Nucleotide sequence of the cDNA for SUSP1 and deduced amino acid sequence of the SUSP1 protein.** The nucleotides are numbered on the right, beginning at the A of the presumed start codon. The amino acid residues are also numbered on the *right*. The *asterisk* indicates the TGA stop codon. (GenBank™ accession number AF196304).

base pair *Sal*I-*Not*I fragment of KIAA0797 was cut off and ligated into the pBluescript II KS(+) vector. Because the clone lacks the 5′-sequence for the N-terminal 28 amino acids of SUSP1, the 5′-anchored PCR method using the 5′-RACE PCR kit (Life Technologies, Inc.) was employed to clone the ultimate 5′-end. The reactions were performed according to the manufacturer's instructions, and a 600-base pair product was obtained. The resulting plasmid containing the full-length cDNA was referred to as pBS/SUSP1. Several independent clones were sequenced on both DNA strands using an automated DNA sequencer to exclude any PCR error, and all restriction sites used for subcloning were verified as well.

To generate the plasmids expressing Ubl·β-galactosidase fusions, the *Spe*I restriction site was introduced immediately upstream of the ATG start codon for Ub in pACUb·β-galactosidase (14) by PCR-based site-directed mutagenesis. The restriction sites for *Spe*I and *Bam*HI were also introduced by PCR to the 5′- and 3′-regions of cDNAs for Ubls, respectively. The PCR products were cut out by *Spe*I and *Bam*HI and ligated into pACUb·β-galactosidase that had been treated with the same restriction enzymes. The plasmids expressing Fub·β-galactosidase, Smt3·β-galactosidase, and SUMO-1·β-galactosidase were gifts from by Drs. Rohan T. Baker (Australian National University), Erica S. Johnson (Rockefeller University), and Rohit Mahajan (Scripps Research Institute), respectively.

Preparation of Purified SUMO-1·PESTc and Smt3·PESTc—To obtain SUMO-1 and Smt3 having a C-terminal peptide extension of MHISPPEPESEEEEEHYC (referred to as PESTc) (17), the DNA fragments encoding SUMO-1·PESTc and Smt3·PESTc were synthesized using PCR and ligated into the expression vector pGEX-2T (Amersham Pharmacia Biotech). Extracts were prepared from *Escherichia coli* cells that had been transformed with the recombinant plasmids and loaded onto a glutathione-Sepharose 4B column equilibrated with phosphate-buffered saline. After washing with the same buffer, the column was treated with thrombin to cleave off glutathione *S*-transferase (GST) that is fused to the N termini of SUMO-1·PESTc and Smt3·PESTc. The proteins were then eluted with the same buffer and subjected to gel filtration on a Sephadex G-75 column to remove thrombin.

Expression of SUSP1 in E. coli—The *E. coli* JM109 cells were transformed with pBS/SUSP1 and grown to a late exponential phase at 37 °C

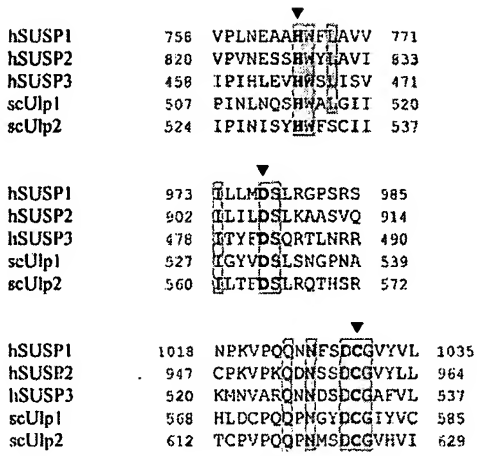

FIG. 2. **Sequence alignment of conserved putative active site residues in SUSPs.** The *arrowheads* indicate the amino acid residues of the catalytic triad (His, Asp, and Cys) in human SUSP1 (*hSUSP1*, this work), putative human SUSPs (*hSUSP2* and *hSUSP3*, GenBank™ accession numbers AF199458 and AF199459, respectively),[2] and *Saccharomyces cerevisiae* Ulps (*ScUlp1* and *ScUlp2*) (12). Of these, hSUSP1 corresponds to "HsUlp1," whose partial sequence was reported by Li and Hochstrasser (12). Identical amino acid residues are denoted by *shading*.

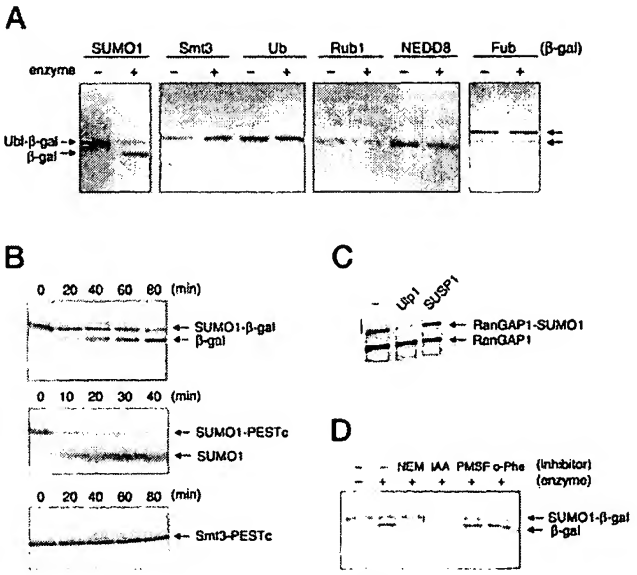

FIG. 3. **Hydrolysis of various Ubl-β-galactosidase fusions, Ubl-peptides, and RanGAP1-SUMO-1 conjugates by SUSP-1.** *A*, hydrolysis of various Ubl-β-galactosidase fusions. The extracts (50 μg) obtained from *E. coli* MC1000 cells expressing each of the Ubl-β-galactosidase fusions were incubated in the absence (− *lanes*) and presence of 20 μg of the partially purified SUSP1 (+ *lanes*) for 2 h at 37 °C. After incubation, the samples were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis using an anti-β-galactosidase antibody. *B*, time course for hydrolysis of SUMO-1-β-galactosidase, SUMO-1-PESTc, and Smt3-PESTc. The SUSP1 enzyme preparation (20 μg) was incubated with the extracts containing SUMO-1-β-galactosidase (50 μg), purified SUMO-1-PESTc (5 μg), or Smt3-PESTc (5 μg) for various periods. The cleavage of SUMO-1-β-galactosidase was assayed as above, and that of SUMO-PESTc and Smt3-PESTc was assayed by staining the gels with Coomassie Brilliant Blue R-250. The *numbers* on top of each gel indicate the incubation period. *C*, hydrolysis of RanGAP1-SUMO-1 conjugates. The extracts containing SUSP1 or Ulp1 and GST-SUMO-1-modified ^{35}S-RanGAP1 were prepared as described under "Experimental Procedures." Reaction mixtures (total 36 μl) containing 15 μl of the enzyme preparations and 4 μl of the labeled substrate were incubated for 3 h at 37 °C. The substrate alone was also incubated as a control (− *lane*). The samples were then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. *RanGAP1-SUMO-1* and *RanGAP1* indicate GST-SUMO-1-modified ^{35}S-RanGAP1 and ^{35}S-RanGAP1, respectively. *D*, effects of various protease inhibitors on the hydrolysis of SUMO-1-β-galactosidase. The SUSP1 enzyme preparation (20 μg) was incubated in the absence (− *lanes*) or presence of 5 mM *N*-ethylmaleimide (*NEM*), iodoacetamide (*IAA*), phenylmethylsulphonyl fluoride (*PMSF*), or *o*-phenanthroline (*o-Phe*) at 37 °C for 10 min. After incubation, the samples were further incubated with the extract containing SUMO-1-β-galactosidase for 2 h at 37 °C. They were then subjected to immunoblot analysis as above.

in Luria broth containing ampicillin. The cultures were then treated with isopropyl-thio-β-*D*-galactoside to 2 mM and further incubated for the next 3 h. After incubation, the cells were collected and resuspended in 25 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol. They were then disrupted by the French press at 14,000 p.s.i. and centrifuged at 100,000 × *g* for 2 h. The resulting supernatants were chromatographed on a DEAE-Sepharose column equilibrated with the same buffer. After washing the column, the bound proteins were eluted with a linear gradient of 0–0.3 M NaCl. Aliquots of the fractions were assayed for the SUSP1 activity on SUMO-1-β-galactosidase fusion (see below), and the fractions with high activity, which eluted at about 0.2 M NaCl, were pooled and used for further studies.

In Vitro Assay for SUSP1 Activity—To assay the activity of SUSP1 *in vitro*, extracts were prepared as described above from the *E. coli* MC1000 cells transformed with the plasmids expressing Ubl-β-galactosidase fusions. The partially purified SUSP1 (20 μg) from the DEAE-Sepharose chromatography was then incubated for 2 h at 37 °C in the absence and presence of the extracts containing Ubl-β-galactosidase fusions (50 μg), purified SUMO-1-PESTc (5 μg), or Smt3-PESTc (5 μg). Reaction mixtures in total volumes of 50 μl also contained 100 mM Tris-HCl, (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. After incubation, the reaction was terminated by adding 20 μl of 10% (w/v) SDS and subjected to polyacrylamide gel electrophoresis under denaturing conditions (15). To determine the cleavage of Ubl-β-galactosidase, immunoblot analysis was performed using an anti-β-galactosidase antibody. For assaying the cleavage of SUMO-1-PESTc and Smt3-PESTc, the gels were directly stained with Coomassie Brilliant Blue R-250.

To assay the activity of SUSP1 on RanGAP1-SUMO-1 conjugates, GST-SUMO-1-modified ^{35}S-RanGAP1 was synthesized using rabbit reticulocyte as reported previously (12). The activity of SUSP1 as well as of yeast Ulp1 was then determined essentially the same as described previously (13). The extracts containing SUSP1 and Ulp1 were prepared from *E. coli* JM109 cells transformed with pBS/SUSP1 and pBS/Ulp1 by following exactly the protocol of Li and Hochstrasser (12) to assay them under the same conditions. After the reaction, the samples were subjected to SDS-polyacrylamide gel electrophoresis on 4–20% gels followed by autoradiography.

Localization of Green Fluorescent Protein (GFP)-SUSP1 Fusion Protein—A DNA construct for GFP-SUSP1 fusion was made by inserting the SUSP1 cDNA to the 3′-end of the open reading frame for GFP using the pEGFP-C1 vector (CLONTECH). The recombinant vector was transiently transfected into NIH3T3 and HeLa cells by LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Cells were grown on glass coverslips coated with 0.1% gelatin and fixed in 3.7% paraformaldehyde at room temperature for 10 min. After fixation, they were washed three times with phosphate-buffered saline, mounted in a vectashield (VECTOR), and examined using a confocal microscope (Carl Zeiss LSM 410).

[2] S.-J. Choi, Y.-J. Jeon, K. I. Kim, S. Nishimori, T. Suzuki, S. Uchida, N. Shimbara, K. Tanaka, and C. H. Chung, unpublished data.

RESULTS AND DISCUSSION

Cloning of a cDNA Encoding SUSP1—The open reading frame specifying a human SUMO-1-specific protease was identified and designated as SUSP1. Baker *et al.* (16) have recently suggested the nomenclature for human Ub-specific protease as USP. By adopting the nomenclature system, we named this putative SUMO-1-specific protease as SUSP1. The 5′-end of the cDNA for SUSP1 was obtained through the 5′-anchored PCR technique using the KIAA0797 cDNA clone, which lacks the 5′-sequence for the N-terminal 28 amino acids of SUSP1. The

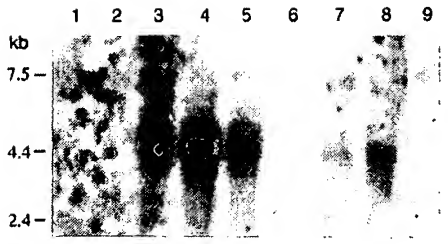

FIG. 4. **Expression of SUSP1 mRNA in various human tissues.** For RNA blot hybridization analysis, multiple tissue Northern blot filters containing 2 μg of poly(A)$^+$ RNAs from various human tissues (CLONTECH) were hybridized with the full-length ^{32}P-labeled SUSP1 cDNA probe. The cDNA probe (25 ng) was labeled using [α-^{32}P]dCTP and a random primer DNA Labeling kit (version 2, TaKaRa, Japan) as recommended by the manufacturer. After incubation for 1 h at 37 °C, the filters were washed under standard conditions and exposed to x-ray films for autoradiography. *Lane 1*, spleen; *lane 2*, thymus; *lane 3*, prostate; *lane 4*, testis; *lane 5*, ovary; *lane 6*, small intestine; *lane 7*, colon; *lane 8*, peripheral blood leukocyte; *lane 9*, brain.

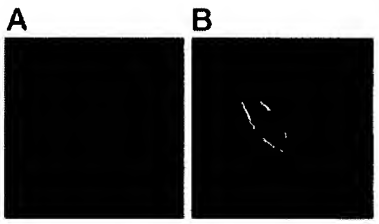

FIG. 5. **Subcellular localization of SUSP1.** NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 1 μg/ml streptomycin, and 10% (v/v) bovine calf serum. They were then plated at 4 × 10^5 cells/ml density on gelatin-coated glass coverslips. After culturing for 24 h, the cells were transfected with the plasmids expressing GFP (*A*) and GFP-SUSP1 fusion (*B*). They were incubated for 48 h, fixed, and observed under a confocal microscope as described under "Experimental Procedures."

position of the ATG start codon was inferred to yield the 3336-base pair open reading frame, which suggest that the full-length cDNA clone encodes a protein of 1112 amino acids with a calculated molecular mass of 126,116 Da and an isoelectric point (pI) of 6.30 (Fig. 1). Like yeast Ulp1 and the related putative enzyme Ulp2 (equivalent to Smt4) (12), SUSP1 as well as the other putative human SUSPs, SUSP2 and SUSP3 (GenBank™ accession numbers AF199458 and AF199459, respectively),[2] had well conserved residues of the catalytic triad (His, Asp, and Cys) and an invariant Gln residue predicted to help to form the oxyanion hole in the active site (Fig. 2). However, the sequence similarity was largely restricted to the conserved active site domains. In addition, SUSP1 showed no evident amino acid sequence similarity to any known deubiquitinating enzymes. On the other hand, it has recently been reported that the partial peptide sequence of an enzyme processing UCRP, a member of Ulps, from human lung carcinoma cells shows a significant similarity to yeast Ub-specific protease, Ubp1 (18). Thus, it is likely that SUSPs described in the present study form a novel family distinct from USPs and UCRP-processing enzymes.

Substrate Specificity of SUSP1—To determine whether SUSP1 is capable of cleaving the carboxyl side of the C-terminal Gly-Gly residues of SUMO-1, the partially purified enzyme was incubated with SUMO-1-β-galactosidase fusion as a model substrate. We also examined whether the enzyme preparation could cleave other Ubl-β-galactosidase fusions. As shown in Fig. 3*A*, SUSP1 efficiently released SUMO-1 from SUMO-1-β-galactosidase. However, it showed little or no activity on any other Ubl-β-galactosidase fusions, including Smt3-β-galactosidase. It also could not release free Ub from Ub-β-galactosidase. To confirm the ability of SUSP1 in cleaving SUMO-1-β-galactosidase, the enzyme was incubated with the substrate as above but for varying periods. As shown in Fig. 3*B* (*top panel*), SUSP1 cleaved SUMO-1-β-galactosidase in a time-dependent fashion.

Smt3 is a yeast homolog of mammalian SUMO-1 (6, 7). In addition, it has been demonstrated that yeast Ulp1 is capable of cleaving Smt3-hemagglutinin fusion as well as SUMO-1-hemagglutinin (12). Because the Smt3 precursor has a much smaller C-terminal extension than Smt3-β-galactosidase, we examined whether SUSP1 might be able to release Smt3 from its fusion with a small peptide, such as Smt3-PESTc. As a control, we also used SUMO-1-PESTc as a substrate. Fig. 3*B* shows that SUSP1 cleaves SUMO-1-PESTc in a time-dependent fashion (*middle panel*) but not Smt3-PESTc at all (*bottom panel*). These results suggest that SUSP1 shows a tight substrate specificity for interaction only with SUMO-1.

We then examined whether SUSP1 might be capable of releasing SUMO-1 from RanGAP1-SUMO-1, using GST-SUMO-1-modified ^{35}S-RanGAP1 as a substrate, in which GST-SUMO-1 is conjugated to RanGAP1 through an isopeptide linkage. Whereas yeast Ulp1 efficiently cleaved GST-SUMO-1-modified RanGAP1 in accord with the earlier report (12), little or no hydrolysis of the substrate was observed with SUSP1 (Fig. 3*C*). Thus, SUSP1 appeared unlikely to process RanGAP1-SUMO-1. However, the lack of the isopeptidase activity of SUSP1 could not be rigorously concluded from the negative *in vitro* result. The enzyme might not be properly folded under the conditions it was expressed or may require modification or interaction with other factors in human cells to act on the RanGAP1p-SUMO-1 conjugate.

Like Ulp1 and Ulp2 in yeast (12), SUSP1 contains one conserved cysteine residue in the putative active site domain (see Fig. 2). Therefore, we examined whether sulfhydryl-blocking reagents could prevent the enzyme activity. Cleavage of SUMO-1-β-galactosidase was completely blocked by incubation of the partially purified SUSP1 with 5 mM *N*-ethylmaleimide or iodoacetamide (Fig. 3*D*). On the other hand, little or no inhibition was observed upon treatment with 5 mM phenylmethylsulphonyl fluoride, an inhibitor of serine proteases, or *o*-phenanthroline, a metal chelating reagent. Ub-aldehyde, a specific inhibitor of deubiquitinating enzymes, could not inhibit the SUSP1 activity on SUMO-1-β-galactosidase (data not shown). These results are consistent with SUSP1 being a cysteine protease. This conclusion is supported by the earlier report on yeast Ulp1 (12), which showed by mutagenesis that the suspected Cys residue is crucial and that the enzyme bears significant sequence homology to the adenovirus cysteine protease.

Expression of SUSP1 mRNA in Human Tissues—Northern blot analysis using a human SUSP1 cDNA as a probe detected a single transcript of about 4.4 kilobases in various human tissues. The size of the human SUSP1 cDNA, consisting of 3336 base pairs and a poly(A) tail, corresponded well with the estimated size of the transcript (Fig. 4). Interestingly, expression of the SUSP1 mRNA was the highest in reproductive organs with the order of testis, ovary, and prostate. Peripheral blood leukocyte and colon also expressed SUSP1 mRNA but to relatively less extents. On the other hand, little or no signals were detected from other human tissues, including brain, liver, lung, kidney, pancreas, spleen, thymus, heart, and skeletal muscle under the same experimental conditions (data not shown), despite the fact that the original cDNA clone for SUSP1 was obtained from human brain. However, we could detect faint signals in brain and small intestine but not in others upon prolonged exposure of the same mRNA blots. Thus, it appears that expression of the SUSP1 mRNA is tissue-specific, partic-

ularly in reproductive organs. These results suggest that SUSP1 may play an important role in reproductive processes.

Subcellular Localization of SUSP1—To explore the distribution of SUSP1 in cells, the plasmids expressing GFP alone or GFP·SUSP1 fusion were transfected to NIH3T3 and HeLa cells. Upon observation of the cells under a confocal microscope, control GFP was found in both the nucleus and the cytoplasm of NIH3T3 cells, as typical small proteins, like GFP, diffuse into nearly all of subcellular organelles (Fig. 5*A*). On the other hand, the GFP·SUSP1 fusion protein was almost exclusively found in the cytoplasm (Fig. 5*B*). Similar data were obtained with HeLa cells (data not shown). Thus, it appears likely that SUSP1 functions primarily in the cytoplasm.

RanGAP1 is targeted from the cytoplasm to the nuclear membrane upon modification by SUMO-1, thus playing an essential role in nuclear transport of proteins (10). SUMO-1 is also involved in negative regulation of the nuclear factor-κB pathway by being conjugated to IκBα, thus preventing ubiquitination of IκBα and nuclear translocation of nuclear factor-κB from the cytoplasm (11). In addition, it has recently been demonstrated that SUMO-1 modification is involved in a variety of other cellular processes, including activation of the transcriptional response of p53 tumor suppressor (19, 20), targeting of the homeodomain-interacting protein kinase 2 to nuclear dots (21), and regulation of septin ring dynamics during cell cycle (22, 23).

The 30-kDa SUMO-1 hydrolase from bovine brain (13) as well as yeast Ulp1 (12) have been shown to release SUMO-1 from RanGAP1·SUMO-1 conjugates. Although in the present study we could not demonstrate the isopeptidase activity of SUSP1 on RanGAP1·SUMO-1, it is possible that SUSP1, located almost exclusively in the cytoplasm, may act on SUMO-1-modified IκBα as well as on other SUMO-1-modified proteins that have not yet been identified.

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